METABOLISM OF NICOTINE IN THE HUMAN AND EXCRETION OF PYRIDINE COMPOUNDS BY SMOKERS

EDWARD R. BOWMAN, LENNOX B. TURNBULL AND HERBERT McKENNIS, JR.

Department of Pharmacology, Medical College of Virginia, Richmond

Reprinted from THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 127, No. 2, October, 1959
Printed in U.S.A.

METABOLISM OF NICOTINE IN THE HUMAN AND EXCRETION OF PYRIDINE COMPOUNDS BY SMOKERS[1]

EDWARD R. BOWMAN,[2] LENNOX B. TURNBULL AND HERBERT McKENNIS, JR.

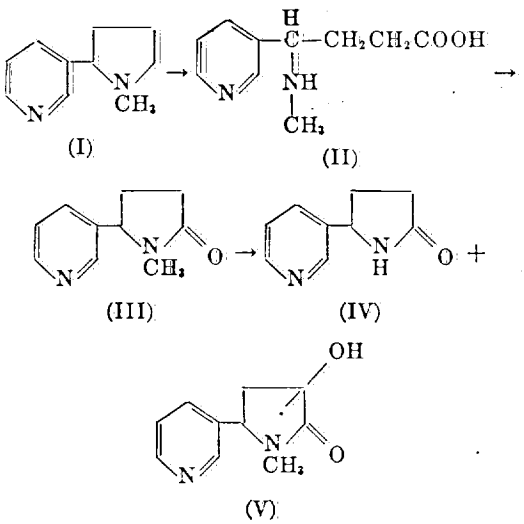
Department of Pharmacology, Medical College of Virginia, Richmond

Received for publication April 7, 1959

On many occasions, such as in industrial accidents or inadvertent overexposure to insecticides, in diagnostic procedures for diabetes insipidus (Chalmers and Lewis, 1951; Cates and Garrod, 1951; Dingman *et al.*, 1957) or in the enjoyment of tobacco smoking, the human assimilates nicotine in varying quantities. It has been frequently observed that following the smoking of tobacco only a small fraction of the absorbed nicotine is eliminated in the urine (Corcoran *et al.*, 1939). It may be deduced, on the basis of animal experiments, that this low excretion of nicotine probably arises from an active metabolism and does not indicate storage or excretion by other pathways.

Studies in the rat (Ganz *et al.*, 1951) and in the dog (Bennett *et al.*, 1954) with uniformly labeled C^{14} nicotine have demonstrated that the major part of the administered radioactivity is eliminated in the urine. In the dog the unchanged nicotine in the urine accounts for approximately 10% of the administered dose.

Examination of the urine of dogs after intravenous administration of *l*-nicotine (I) has led (McKennis *et al.*, 1957–1959) to the isolation and identification of a variety of metabolites including γ-(3-pyridyl)-γ-methylaminobutyric acid (II) and its lactam cotinine (III), desmethylcotinine (IV) and hydroxycotinine (V). γ-(3-Pyridyl)-γ-methylaminobutyric acid can under conditions of physiological pH and temperature (McKennis *et al.*, 1957) spontaneously lactamize to yield cotinine, and the latter gives rise to both desmethylcotinine and hydroxycotinine when administered to the dog (McKennis *et al.*, 1958b, 1959). The known metabolic interrelationships are summarized.



(I) (II) (III) (IV) (V)

The development of the procedures used in the foregoing have made possible a study of nicotine metabolism in the human. This communication describes studies on the isolation of some of the chloroform-soluble metabolites of nicotine present in the urine of the human (non-smoker) and of pyridine compounds present in the urine of human smokers.

METHODS AND MATERIALS. *Administration of nicotine to the human.* A normal adult male (80.4 kg) (non-smoker) received an oral dose of 30 mg of *l*-nicotine per day (in consecutive hourly daytime doses of 3 mg each in gelatine capsules). The experimental period was concluded at the end of the third day because of nausea and the accompaniment of vomiting on the commencement of the fourth day.

Procedure for the isolation of the metabolites. The combined total urine volume (5 liters) from the 3-day period was adjusted to pH 10 with ammonia water and then exhaustively extracted with chloroform. The residue after the evapora-

[1] Presented in part at the 36th Annual Meeting, Virginia Academy of Science, Roanoke, Virginia, May 9, 1958 and at the Twelfth Tobacco Chemists' Research Conference, Durham, North Carolina, October 24, 1958.

[2] Public Health Research Fellow of the National Heart Institute, National Institutes of Health, U. S. Public Health Service.

tion of the chloroform was chromatographed on Whatman no. 1 paper with 0.5 N ammonia water (1 volume)-95% ethanol (1 volume)-n-butanol (4 volumes) (McKennis *et al.*, 1957). The Koenig positive zones were located with a spray of alcoholic *p*-aminobenzoic acid which was followed by treatment with vapors of cyanogen bromide. Paper chromatograms were also prepared with *sec.* butanol-formic acid-water (Hausman, 1952).

The residue from the chloroform extract was dissolved in 25 ml of water and adjusted to pH 2 with 5 N HCl. The solution was passed through Dowex 50 (H^+) (20 × 150 mm). After a water wash the column was eluted with 80 ml of N/1 ammonia water. The ammoniacal solution was concentrated to a brown residue. A solution of the latter in 10 ml of water was adjusted to pH 9 with N/1 ammonia water and passed through Dowex 1 (OH^-) (15 × 100 mm). The effluent and water wash were concentrated to a brown gum (149 mg). Elution of the column with N/1 acetic acid served to remove a component with an R_f value of 0.74 (ammonia-ethanol-butanol). An extract of the gum in 23 ml of chloroform was poured onto an acid-washed alumina column (5 g) and eluted with ether containing successively increasing concentrations of methanol. The fractions, obtained with 1 to 10% methanolic ether, containing a single Koenig component at R_f 0.74 (ammonia-ethanol-butanol), were combined. Upon evaporation of the solvent 10.1 mg of cotinine were obtained as a light colored oil (representing about 10% of the administered nicotine dose), λ max 260 mμ in ethanolic HCl. The oil was converted to 21.5 mg of cotinine dipicrate, micro m.p., 102.5°C.

Fractions with R_f 0.61 (ammonia-ethanol-butanol), obtained from the alumina column (above) with 10 to 100% methanol, were combined and evaporated to give 14.7 mg of gum. The latter, after treatment with acetic anhydride-pyridine and upon evaporation of the reagents, was rechromatographed on alumina as described above. Two fractions R_f 0.61 (1.62 mg) and R_f 0.75 were obtained.

Treatment of smokers' urine. Smokers' urine (60 liters) was obtained by voluntary daytime contributions from male laboratory workers. Chloroform extraction of the urine was accomplished in the previously described manner. The residue from the evaporation of the chloroform was treated with water and methanol to obtain all of the Koenig positive material in solution. Cotinine (87 mg) was isolated and then converted to the dipicrate by the procedure described above.

Examination of human urine for 3-aminomethylpyridines. Samples of urine from smokers or the subject who ingested nicotine were adjusted to pH 2 and placed on Dowex 50 (H^+). The effluent and an ammoniacal eluate of the resin column were treated with cyanogen bromide (Larson and Haag, 1942) before and after boiling.

Preparation of cotinine dipicrate. A solution of synthetic cotinine (500 mg, 0.0029 mol) (McKennis *et al.*, 1959) in 1 ml of methanol, and 1.6 g (0.007 mol) of picric acid was heated to boiling. Upon cooling and scratching, a yellow crystalline precipitate formed. The product had a constant micro m.p. (102.5°C) after 6 recrystallizations from methanol. Calculated for $C_{22}H_{18}N_8O_{15}$: C, 41.65; H, 2.86; N, 17.66. Found: C, 42.37; H, 2.89; N, 17.97.

Cotinine monopicrate from cotinine dipicrate. Cotinine dipicrate was dissolved in a minimum amount of hot absolute alcohol. The solution upon cooling deposited yellow crystals. After 6 recrystallizations the micro m.p. was 104°–105°C. Calculated for $C_{16}H_{15}N_5O_8$: C, 47.41; H, 3.73; N, 17.28. Found: C, 47.30; H, 3.75; N, 17.16.

RESULTS. During the 3-day period the human subject excreted approximately 10% of the administered nicotine in the form of cotinine. Due to the fact that tobacco smokers' urine was obtained only during the daytime and without data on the smoking habits of the subjects or composition of the smoke it is not possible to calculate percentage of metabolism. Cotinine, in addition to other pyridine compounds, has been identified in tobacco smoke (Quin, 1958; Rayburn, personal communication, 1958).

The chromatographic evidence (table 1) points to the presence of cotinine, desmethylcotinine and hydroxycotinine in all samples examined. Positive identification of cotinine was effected by infrared spectrograms of the picric acid salts of cotinine. Double verification was made by converting dipicrates to monopicrates. The dipicrate was identical to a sample prepared in the laboratory of the Japan Monopoly Corporation (Wada *et al.*, 1959). Authentic salts and corresponding isolated salts had the same melting point. The mixed melting points showed no depression.

The cyanogen bromide reaction of Dowex 50 (H^+)-treated urine was negative. The ammoniacal eluate of the resin was positive and gave a pink color. Following heat treatment, which cyclizes γ-(3-pyridyl)-γ-methylaminobutyric acid to cotinine, the pink color was no longer obtained on test samples.

DISCUSSION. The results obtained in the survey of nicotine metabolism in the human indicate a

TABLE I

*R_f values of the chloroform-soluble, Koenig positive components of the urine from the human (non-smoker), human smoker and the dog**

Source	R_f Values	
	Ammonia-ethanol-butanol	*sec*-Butanol-formic acid-water
Human (non-smoker)	0.61§ and 0.74‡	0.21§ and 0.37‡
Human smoker	0.61,§ 0.74,‡ 0.85,† 0.90	0.21§ and 0.37‡
Dog	0.61,§ 0.74,‡ 0.85†	0.21§ and 0.37‡

* Bladder urine collected from dogs (receiving 10 mg/kg nicotine by intravenous infusion over an 8-hr period) via indwelling catheters. Control urines of both species yielded no Koenig positive zones. Nicotine was administered orally to the human subject. Pooled urine from male smokers was employed.

† Cochromatographed with *l*-nicotine.

‡ Cochromatographed with cotinine.

§ Cochromatographed with desmethylcotinine. Fraction acetylated to give components corresponding to acetoxycotinine and desmethylcotinine.

similarity to the previous results obtained in the dog. Chemical isolation of cotinine as one of the metabolites in both species points to a desirability of a study of cotinine metabolism in the human. It has been established that cotinine serves as an intermediate in the degradation of nicotine in the dog. The paper chromatograms of the chloroform-soluble fraction of human urine strongly suggest that cotinine has a similar role in man. Since the desmethylcotinine fraction (R_f 0.61, ammonia-ethanol-butanol) of urine yields on acetylation material with chromatographic characteristics of desmethylcotinine and acetoxycotinine, it is inferred that the pathway of nicotine metabolism in the human may resemble that of dog, *viz.*, nicotine → cotinine → hydroxycotinine plus desmethylcotinine.

Similar pathways are inferred also from a study of the urine of smokers (table 1). Both dog urine and smokers' urine contain material which is Koenig positive at R_f 0.85 (ammonia-ethanol-butanol) corresponding to nicotine. Other investigators have noted the urinary excretion of nicotine under comparable conditions. The human subject who received nicotine excreted, in contrast, no nicotine detectable under the conditions employed for isolation. One explanation for this difference may lie in the differences in route of administration. In both the smoker and the dog receiving nicotine intravenously it is anticipated that some nicotine may be presented to the kidney for excretion without opportunity for the liver (Hucker *et al.*, 1959) or other tissues (Akira, 1957; Miller and Larson, 1953; Werle and Uschold, 1948) to degrade the compound. In contrast, absorption of orally administered nicotine may provide, via portal circulation, a unique opportunity for metabolic transformation in the liver.

The component in smokers' urine at R_f 0.90 (ammonia-ethanol-butanol), which was not encountered in either dog urine or human urine after nicotine, may represent a new nicotine metabolite. On the other hand, pyridine compounds other than nicotine in smoke may be responsible for appearance of the material. It is possible, too, that, unbeknown to us, some of the volunteers may have been receiving medication which is responsible for this component.

Although the present study does not directly concern itself with the immediate precursors of cotinine some conclusions may be drawn with respect to the source of this compound. γ-(3-Pyridyl)-γ-methylaminobutyric acid, which is responsible for a pink color produced by addition of cyanogen bromide to the urine of the nicotine-treated dog, is spontaneously lactamized to cotinine under physiological conditions of pH and temperature (McKennis *et al.*, 1958a). Werle *et al.* (1956) have reported that smokers' urine gives the color reaction with cyanogen bromide. In the present study material obtained, following smoking or ingestion of nicotine, by eluting pyridine components of the urine from Dowex 50 (H^+) showed a similar behavior with cyanogen bromide. The reaction could not be obtained if the material was heated prior to addition of cyanogen bromide. This behavior parallels that of γ-3-pyridyl-γ-methylaminobutyric acid which loses its color-producing ability as the result of lactam formation (acylation of the methylamino group). It may be inferred then that in the human γ-3-pyridyl-γ-methylaminobutyric acid is a nicotine metabolite which lactamizes to cotinine.

Various studies *in vitro* provide evidence for an oxidative metabolism of nicotine to cotinine without necessity for the intermediate methylamino acid. Nicotine in the presence of peroxide or merely on storage (Frankenburg and Vaitekunas, 1957) is oxidized to cotinine. This suggests the possibility of catalase-peroxide catalysis in the formation of cotinine *in vivo*. Thus, at least two pathways to the formation of the important intermediate cotinine, one direct and the other

via γ-(3-pyridyl)-γ-methylaminobutyric acid, are potentially available for physiological disposition of nicotine.

SUMMARY

Following the smoking of tobacco or ingestion of nicotine the urine of human subjects contained cotinine which was identified as the picrate and dipicrate. Chromatograms of the urines suggest the presence of hydroxycotinine and desmethylcotinine and related Koenig positive components and lead to the conclusion that cotinine plays, in man as well as in the dog, an important role as intermediate in nicotine metabolism.

ACKNOWLEDGMENTS. The authors wish to acknowledge generous support by grants from the Tobacco Industry Research Committee and the American Tobacco Company. Dr. E. Wada kindly supplied a sample of cotinine dipicrate for comparison. Mr. J. Scott Osborne, Research Laboratory, the American Tobacco Company, kindly made the infrared determinations.

REFERENCES

AKIRA, T.: Folia pharmacol. Japon. **53:** 553, 1957.

BENNETT, D. R., TEDESCHI, R. E. AND LARSON, P. S.: Arch int. Pharmacodyn. **98:** 221, 1954.

CATES, J. E. AND GARROD, O.: Clin. Sci. **10:** 145, 1951.

CHALMERS, T. M. AND LEWIS, A. A. G.: Clin. Sci. **10:** 137, 1951.

CORCORAN, A. C., HELMER, O. M. AND PAGE, I. H.: J. biol. Chem. **129:** 89, 1939.

DINGMAN, J. F., BENIRSCHKE, K. AND THORN, G. W.: Amer. J. Med. **23:** 226, 1957.

FRANKENBURG, W. G. AND VAITEKUNAS, A. A.: J. Amer. chem. Soc. **79:** 149, 1957.

GANZ, A., KENSEY, F. E. AND GEILING, E. M. K.: THIS JOURNAL **103:** 209, 1951.

HAUSMAN, W.: J. Amer. chem. Soc. **74:** 3181, 1952.

HUCKER, H. B., GILLETTE, J. R. AND BRODIE, B. B.: Nature **183:** 47, 1959.

LARSON, P. S. AND HAAG, H. B.: THIS JOURNAL **76:** 240, 1942.

MCKENNIS, H., JR., TURNBULL, L. B. AND BOWMAN, E. R.: J. Amer. chem. Soc. **79:** 6342, 1957.

MCKENNIS, H., JR., TURNBULL, L. B. AND BOWMAN, E. R.: J. Amer. chem. Soc. **80:** 6597, 1958a.

MCKENNIS, H., JR., TURNBULL, L. B. AND BOWMAN, E. R.: Abstracts of Communications, p. 184, IV Int. Cong. of Biochem., Vienna, Austria, 1958b.

MCKENNIS, H., JR., TURNBULL, L. B., BOWMAN, E. R. AND WADA, E.: J. Amer. chem. Soc., in press, 1959.

MILLER, A. W. AND LARSON, P. S.: THIS JOURNAL **109:** 218, 1953.

QUIN, L. D.: Tobacco Chemists' Research Conference, Durham, N. C., 1958.

WADA, E., KISAKI, T. AND SAITO, K.: Arch. Biochem. Biophys. **79:** 124, 1959.

WERLE, E., SCHIEVELBEIN, H. AND SPIETH, D.: Arztl.-Forsch. **6:** 322, 1956.

WERLE, E. AND USCHOLD, E.: Biochem. Z. **318:** 531, 1948.